MICROBE ASSISTED VALORIZATION OF LIGNIN WASTE

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Certificate

I hereby declare that the short synopsis " **Microbe Assisted Valorization Of Lignin Waste**" being submitted in partial fulfillment of the requirement for the Degree of Doctor of Philosophy at Department of Biotechnology under Faculty of Engineering and Technology of Manav Rachna International Institute of Research & Studies (MRIIRS), Faridabad during the Academic Year 2016-17

It is a bona fide record of original work carried out under the Supervision of Dr. Sarita Sachdeva, Professor, Biotechnology, MRIIRS, Faridabad and under the Joint supervision of Dr. Manoj Kumar, Senior Research Manager (SRM), IOCL R&D Center, Faridabad and has not been submitted in full or part to any other university or institute for degree or diploma. Further, it is certified that short synopsis is plagiarism free and proof has been submitted.

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ABSTRACT

Lignin is complex in nature due to its heterogeneity in polymer structure made up of aromatics and is part of most terrestrial plants providing structural integrity. Approximately, it constitutes in a range of 15 to 40% dry weight. Several studies reported have been utilizing the lignin containing biomass as feedstock for ethanol production. However, the major limitation of the established process is disposal of residual lignin enriched stream, which contains recalcitrant complex carbon moieties that has calorific value but unable to tap it sustainably. Various physico-chemical and thermal processes were attempted to break down the lignin rich residual waste, obtained from biomass derived fuels, but were not energy efficient as well as not eco-friendly making the whole process unsustainable. At this juncture, researchers across the globe are in search of alternate biological routes to valorize the lignin rich waste along with the other components of the biomass. The three potential possible biological routes for the depolymerization of lignin containing waste include enzymatic, fungal and bacterial routes which are cost effective, energy efficient and also eco-friendly. Among the three biological routes, fungal and enzymatic routes are well studied but both of them have their own limitations. They cannot with stand at high temperature, pH and substrate conditions. In this context, the present project was aimed to develop microbe assisted process for valorization of lignin containing waste towards biogas production. The main objective of the proposed project includes the breakdown of complex lignin molecules by selectively enriched microbial consortium and its further conversion to biogas production. Outcome of this study may open new avenues in the lignin valorization research and also helps in overcoming some of the existing major limitations of the biomass derived fuels.

Key words: Lignin degradation, bioremediation, biomass valorization, biofuels, bioaromatics

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1. Introduction

Lignin is complex in nature due to its heterogeneity in polymer structure made up of aromatics and is part of most terrestrial plants providing structural integrity. Lignin constitutes approximately 15 to 40% (%dry weight basis). Most of the large scale industries processing plant polysaccharides, in general, burns the lignin content to generate power through which the productive biomass transformation is balanced. Advent of biorefineries that generate liquid transportation fuels from cellulosic biomass, generates significantly higher lignin content than that is necessary to supply the required power for the total operation. It is therefore need of the hour for transforming it to value-added products and the efforts are underway. The phenylpropanoid units cross linked extensively constitute lignin and also make it resistant to microbial degradation. Also, lignin composition varies from plant to plant. Considerable interest has been generated in lignin valorization/degradation, due to the fact that it is the major hurdle in the conversion of lignocellulosic biomass to biofuels and chemicals (Lee 1997). After the utilization of cellulosic part of biomass in biorefinery processes, the residual waste is rich in lignin component which can be utilized as C-source. Fungi are known for lignin biodegradation, especially the white and brown rot fungi, due to the release of extracellular oxidative enzymes. On the contrary, microbes need to be evaluated for their potential to degrade lignocelluloses,considering their robust environmental adaptability as well as metabolic diversity. Some of the bacteria can degrade lignin and assimilated. In this context, the proposed study evaluates the feasibility of microbial lignin degradation as well as its transformation to biofuels. This proposal is aimed for the development of integrated process for converting the low value lignin into high value fuel moieties and also helps in complementing the existing biorenery processes.

2. Literature review

Biomass is being considered as most significant renewable energy resource, accounting for 75% of rural energy needs, where more than 70% of Indian population is of rural background. Two major biomass sources identified in India, viz., Agricultural residues and forest & wasteland residues, accounts for about 145105 and 104048 KT/annum respectively and are equivalent to 18730 and 14561 MWe (Biomass Knowledge portal). Energy

requirements of the domestic as well as industrial applications are met by the forest trees and this is affecting the sustainable utilization of wood. Agriculture biomass can be classified into two groups, viz., field based biomass that includes residual plant material after main crop harvest (for example, straw, stalks, leaves, roots, branches, etc) and process based biomass including agro industry residues after cleaning, threshing, sieving, crushing, etc. The byproducts of processes after harvest including husk, dust, or straws, groundnut shells, bagasse, rice husk, coconut shell, corn cobs, etc., also comes under available biomass waste (Biomass Knowledge portal). The lignin content of several biomass sources has been given in Table 1. Biomass has not been placed in appropriate place in the India's energy balance, *in spite* of meeting major portion of required energy. This is due to insufficient availability of documented data for the biomass availability, consumption and utilization pattern as well as its adaptability (Mukherjee et al., 2016).

On the other hand, there are certain technologies being evolved for the biomass valorization, such as ethanol fermentation but the challenge remained was the lignin content. Lignin is aromatic heteropolymer providing strength and rigidity to terrestrial plants as well as facilitating the water and nutrient transport to the plant tissues. In general, lignin is composed of *p*-coumaryl, coniferyl and sinapyl alcohol moieties. Also, the hydroxycinnamic acid moieties contribute to significant extent in agricultural residues resulting in strong heterogeneity of lignin (Bonawitz et al., 2014; Wagner et al., 2015; Eudes et al., 2014; Wilkerson et al., 2014; Beckham et al., 2016). Lignin valorization has become essentially important as the effluent after ethanol production got enriched lignin content and it also needs to be treated prior to disposal as per the environmental guidelines. Moreover, the lignin waste stream also has substantial amount of carbon remaining to be utilized as energy source (Davis et al., 2013). Most of the current practices of lignin valorization include generating power and heat through combustion. Cellulosic ethanol plants are being commissioned across the globe and the major hurdle for the success of these plants has been identified as the lack of broader lignin valorization technologies. Typical bioethanol plant of 2000 ton capacity using corn stover will produce approximately 70,000 tons per annum (Davis et al., 2013).

Table 1: Comparative lignin component present in various biomass sources (on dry weight basis) (Sun and Cheng, 2002)

Biomass	**Lignin component (%)**	**Cellulose component (%)**	**Hemicellulose component (%)**
Wheat straw	15	30	50
Hardwoods stems	18–25	40–55	24–40
Cotton seed hairs	0	80-95	5-20
Nut shells	30-40	25-30	25-30
Corn cobs	15	45	35
Grasses	10-30	25-40	35-50
Softwood stems	25-35	45–50	25-35
Paper	0-15	85-99	0
Leaves	0	15-20	80-85
Newspaper	18-30	40-55	25-40
Sorted refuse	20	60	20
Waste papers from chemical pulps	5-10	60-70	10-20
Wheat straw	8.9-17.3	32.9-50	24-35.5
Coastal Bermuda Grass	6.4	25	35.7
Primary wastewater Solids	24-29	8-15	n.a*
Swine waste	n.a	6	28
Solid cattle manure	2.7-5.7	1.6-4.7	1.4-3.3
Switchgrass	12	45	31.4
Sugarcane bagasse	10-20	40-41.3	27-37.5
Rice straw	9.9-24	36.2-47	19-24.5

***n.a, not available**

Similarly, paper and pulp industry produce millions of tons of lignin based wastewater, which is further expected to increase due to the ongoing research developments towards replacing fossil fuels (De los Santos Ramos et al., 2009). On the other hand, biorefinery processes utilize the cellulosic part and the residual waste rich in lignin can be considered as potential feedstock (Stewart, 2008; Zaldivar et al., 2001; Ragauskas et al., 2006; Himmel et al., 2007). Though, huge amount of lignin rich waste available, the commercially exploited kraft lignin stands less than 100000 tonnes/annum (Gosselink et al., 2004). Instead of losing the lignin potential in the form of power and heat, it can used as feedstock for producing value added chemicals (Zaldivar et al., 2001; Ragauskas et al., 2006).

Significant efforts have been put in for the lignin valorization, from decades, to breakdown the lignin and upgrade it to fuels or chemicals through catalytic, thermal, and biological approaches (Zakzeski et al., 2010; Ragauskas et al., 2014). Controlled depolymerization of lignin can add commercial value but is stalled by its high resistance for breakdown through chemical as well as biological degradation (Martinez et al., 2005). Lignin can be depolymerized via thermo-chemical methods such as pyrolysis/ gasification, oxidation by chemicals, hydrolysis or hydrogenolysis, etc (Pandey and Kim, 2011). However, all these processes are not environmentally benign, and are energy intensive as well as produce secondary waste (Ward and Singh, 2002) and therefore cannot be considered under the sustainable perspective.

Most of the lignin depolymerization methods result in production of diverse heterogeneous aromatic compounds, with very few exceptions, and the product profile normally depends on the nature of feedstock and the method used for degradation. Preliminarily, lignin is degraded by the aromatic radicals generated by oxidative enzymes like peroxidases, laccases, etc., from fungi or bacteria (Martinez et al., 2010; Martinez et al., 2010). These enzymatic reactions produce diverse group of oxygenated aromatics. In brief, the typical bioconversion methods employ milder thermo-chemical pre-treatment coupled with enzymatic hydrolysis, which converts the polysaccharides into soluble sugars that can be further converted to ethanol, leaving the lignin rich streams as waste (Chundawat et al., 2011). Further upgradation of lignin enriched streams is possible either from pretreatment (upstream), or from hydrolysis of polysaccharides (downstream). It is noteworthy to understand that

complete fractionation of lignin from biomass source as such is challenging, especially without affecting adversely the yields of carbohydrate.

Presently, diverse options for biomass pretreatment are available and among them few are capable of degrading lignin partly but the others are not. At industrial scale, the methods adapted till now include, hydrothermal, acid and ammonia based, etc., while few other approaches are moving towards scale up (pilot or demonstration). On similar note, few bench scale methods like ionic liquids or solvent-based fractionations, etc., are also being pursued, that can fractionate lignin selectively from whole biomass (Socha et al., 2014). Reductive Catalytic Fractionation (RCF) is another emerging approach that combines active redox catalyst with biomass under reduction environment for cleavage of β-O-4 linkages selectively (Ferrini and Rinaldi, 2014; Van den Bosch et al., 2015). On the other hand, pretreatment approaches under high pH using bases like NaOH, are also able to degrade the lignin fraction partially resulting in lignin rich waste streams (Chen et al., 2012; Karp et al., 2015).

On the contrary, the solubilized or residual lignin obtained after pretreatment/ fractionation or after hydrolysis step will normally be a solid and is contaminated with catalyst originated from biological and/or chemical nature, which also depends on the method of processing. The solid lignin stream needs to be depolymerized to simpler molecules to be acquiescent for biological degradation. Various approaches have been proposed over last century for the lignin breakdown but are not established with encouraging results due to the fact that the breakdown of labile C–O linkages and recalcitrant C–C linkages making the residual lignin not susceptible. Fungi were reported to depict efficient lignin breakdown but has the limitation pertaining to their stability under extremities (Hatakka, 1994). On contrary, bacteria can be considered as potential candidates for lignin degradation due to their immense environmental adaptability and biochemical versatility. Few bacteria were reported in literature for lignin degradation, viz., *Streptomyces viridosporus* T7A (Ramachandra et al., 1988), *Pseudomonas paucimobilis* SYK-6 (Katayama et al., 1988), *Azotobacter, Bacillus megatarium, Serratia marcescens* (Morii et al., 1995), *Aneurinibacillus aneurinilyticus* (Raj et al., 2007), *Pseudomonas putida mt-2, Rhodococcus jostii RHA1, Pseudomonas putida* KT2440 (Linger et al., 2014) *Pseudomonas fluorescens* (Salvachu et al., 2015), *Rhodococcus jostii* RHA1 (Salvachu et al., 2015), *Rhodococcus opacus* (Kosa et al., 2013), *Acinetobacter*

baylyi ADP1 (Barbe, 2004), *Amycolatopsis* sp. 75iv2 (Salvachu et al., 2015), *Sphingomonas* sp. strain SYK-6 (Masai et al., 2007) etc. Few studies also showed the screening and isolation of lignin degrading microbial consortium from different mixed culture sources, viz., termite guts (Geib et al., 2008), reeds pond sludge (Wang et al., 2013), environmental samples like woodland soil, composted wheat straw (Taylor et al., 2012) etc. *P. Putida* KT2440 depicted significant potential as biocatalyst in transforming lignin into aromatic compounds, till date. Irrespective of the source and group of microbe, the ideal lignin degrading microbe should have some specific characteristics, viz., aromatic substrate specificity, genetic tractability, tolerant to stress, and ability to work in industrial bioreactors. Enzymes can also be considered for efficient and specific lignin breakdown, especially under ambient conditions, with low energy input and without any environmental impact (Perez et al., 2002; Sun and Cheng, 2002). However, structural modifications of lignin during lignocellulose processing is the major limitation (Crawford, 1981; Emmel et al., 2003) and hence, the industrial lignin waste cannot be effectively degraded in controlled manner using ligninolytic enzyme systems (Bandounas et al., 2011). In this context, the proposed project consider using lignin containing biomass as potential substrate for biogas production, selection of suitable microbes, strain development through engineering and integrated process development as major options to study.

3. Description of broad area

Several studies reported have been utilizing the lignin containing biomass as feedstock for ethanol production. However, the major limitation of the established process is disposal of residual lignin enriched stream, which contains recalcitrant complex carbon moieties that has calorific value but unable to tap it sustainably. Physico-chemical and thermal processes used as integration to biotechnological processes of biomass derived fuels, making the whole process unsustainable and non eco-friendly. At this juncture, researchers across the globe are in search of alternate biological routes to valorize the lignin along with the other components of the biomass. The main objective of the proposed project includes the valorization of lignin molecules also along with the other components of biomass towards biogas production. Outcome of this study may open new avenues in the lignin valorization research and also helps in overcoming the existing major limitation of the biomass derived fuels.

4. Motivation of the Study

Lignin is the second abundant constituent found in lignocellolosic biomass. Currently, efforts are being made to convert cellulose and hemicelluloses of lignocellosic biomass to ethanol, however, no efforts are being made for residual lignin. Hence, after ethanol production from lignocelulosic biomass lignin remained as waste which is normally burned and used for generating heat energy or chemically breakdown to its constituents/simpler molecules and subjected to other energy generation processes. Conversion of lignin in to valuable chemicals is a challenge because of its complex structure and high carbon content. The major limitation for the chemical and thermal process used for lignin degradation is high energy consuming and non eco-friendly. To overcome this problem there is a need to develop cost effective and environ friendly biological methods for the proper conversion of lignin components in to valuable products. In this direction, current work is an attempt towards valorization of lignin rich waste coming from ethanol fermentation via biological route to produce biogas or other energy molecules.

5. Objectives of Research

The proposed objectives of the research are as follows

1. Selective enrichment of lignin degrading microbial consortia and detailed characterization
2. Identification of genes responsible for lignin degradation and their expression level in the enriched microbes.
3. Process optimization for microbially catalyzed lignin containing waste degradation
4. Characterization of biologically degraded lignin residue and screening for its bioactivity, if any
5. Integrated approach for lignin degradation and biogas production

6. Methodology to be adopted

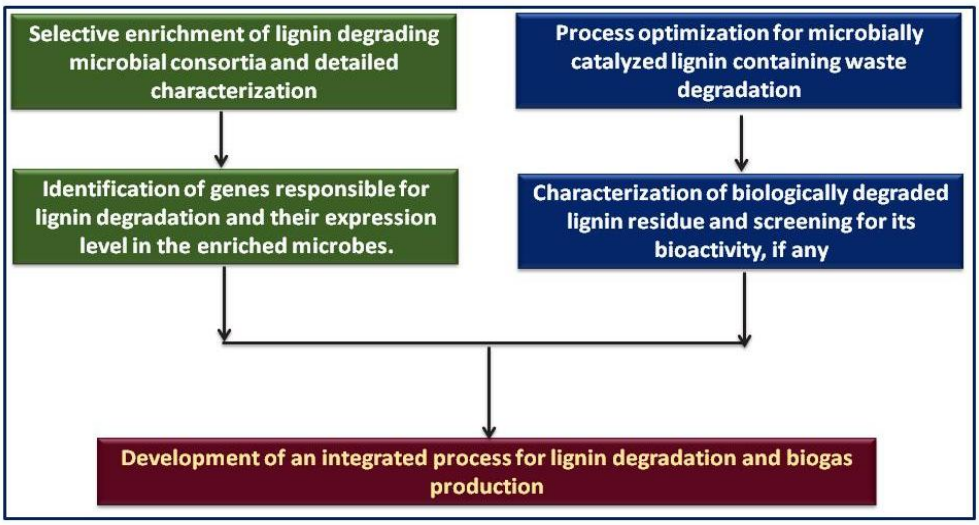
Selective enrichment of lignin degrading microbial consortia and detailed characterization
Process optimization for microbially catalyzed lignin containing waste degradation
Identification of genes responsible for lignin degradation and their expression level in the enriched microbes.
Characterization of biologically degraded lignin residue and screening for its bioactivity, if any
Development of an integrated process for lignin degradation and biogas production

Figure 1: Step-wise experimental approach for the proposed study

1. Selective enrichment of lignin degrading microbial consortia and their characterization

   Mixed culture from different sources, viz., anaerobic sludge from industrial ETP, PAH contaminated site, land filling sites of municipal solid waste (MSW), will be collected for enrichment and isolation of microbes that are capable of utilizing lignin as C-source. Isolation of microbes will be done using routine microbiological methods and their Genus level identification of will be done using molecular techniques like, PCR, DGGE, band elution and DNA sequencing.

2. Identification of genes responsible for lignin degradation and their expression level in the enriched microbes.

   Primers will be designed for genes related to lignin degrading pathway and their expression level in isolated potential microbial strains. Strain improvement will be carried out through random mutagenic studies and genome shuffling to increase the relevant gene expression of the wild strains. Relative improvement in the expression level of gene in the mutated strains against wild strain will be studied.

3. Process optimization for microbially catalyzed lignin containing waste degradation

   Lignin degradation potential of isolated and enriched culture will be studied. Further, optimization of various operating parameters, like, pH, retention time,

oxygen availability, etc., for higher lignin degradation will be optimized. The optimum operating conditions will be obtained through application of statistical tools. Augmentation of the highly efficient mutated strains onto the enriched mixed culture will be done to increase the biogas production ability from lignin degradation.

4. Characterization of biologically degraded lignin residue and screening for its bioactivity, if any

   Detailed characterization of the lignin containing biomass will be done before and after utilizing it for the biogas production. The breakdown products of the lignin will be assessed both qualitatively and quantitatively using standard protocols. The residual lignin will also be screened for any biological activity against different groups of bacteria.

5. Integrated approach for lignin degradation and biogas production

   Bioreactor of 2L scale will be operated for lignin degradation coupled to biogas production under optimized conditions using enriched biocatalyst. Data will be collected at this stage towards up-scaling of the process and also to draw conclusions with respect to the biogas production potential of lignin containing biomass as feedstock.

7. Proposed/expected outcome of the research

Major expected outcome of the proposed project is an optimized, efficient and sustainable bio-process for enhanced microbial degradation of lignin containing waste coupled to harnessing biogas.

Specific deliverables of the project are as follows:

1 Selectively enriched/improved microbial consortia having ability to degrade lignin

2 Optimized process for higher lignin degradation and its transformation to fuel moieties

3 Integrated process for converting lignin into biogas/ biofuels

Outcome of this study may open new avenues in the lignin valorization research and also helps in overcoming the existing major limitation of the biomass derived fuel

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